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Functional Significance of Manganese Catalase in *Lactobacillus plantarum*

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A strain of *Lactobacillus plantarum* which was unable to produce manganese (Mn)catalase (ATCC 8014) grew somewhat more rapidly and to a slightly higher plateau density than did an Mn catalase-positive strain (ATCC 14421), and this was the case during aerobic or anaerobic growth. However, when maintenance of viability was measured during the stationary phase of the growth cycle, the advantage provided by Mn catalase was obvious. Thus, the viability of ATCC 14431 was undiminished over 21 h of aerobic incubation, during the stationary phase, whereas that of ATCC 8014 decreased by seven orders of magnitude. Addition of catalase to the medium or growth in the presence of hemin, which allows catalase synthesis, protected ATCC 8014 against this loss of viability. Suppression of Mn catalase within ATCC 14431 by treatment with NH_2OH caused the cells to lose viability when exposed to 4 mM H_2O_2.

Lactobacillus plantarum exhibits several peculiarities in its defenses against oxygen toxicity. In this organism divalent manganese (Mn) is accumulated to an intracellular level of approximately 25 mM and serves as a functional replacement for the superoxide dismutases found in most cells (1–4). Moreover *L. plantarum*, like certain pediococci, leuconostocs, and streptococci, is unable to synthesize heme and, when grown in the absence of heme, produces a hemeless catalase which has been called pseudocatalase (7, 11–14). We recently reported the isolation of the pseudocatalase and its characterization as a hexameric manganienzyme (14a), hereafter called Mn catalase.

One can assume that the Mn catalase is important to the organisms which produce it, but this has not heretofore been demonstrated. Moreover, the available literature leaves this question in doubt. Thus, Mn catalase-positive strains of pediococci exhibited a growth advantage during aerobic culture on glycerol (8), but a comparable advantage was not noted with either *Streptococcus faecalis* or *L. plantarum* whether grown on glycerol or glucose (14). Indeed, Mn catalase-negative strains of *L. plantarum* were reported to grow more rapidly than Mn catalase-positive strains under either aerobic or anaerobic conditions (17).

Since an Mn catalase-negative strain of *L. plantarum* accumulated H_2O_2 during the late log and stationary phases, whereas an Mn catalase-positive strain did not (14a), it appeared possible that measurements of retention of viability might expose the importance of the Mn catalase. Another approach would be to inactivate the Mn catalase within *L. plantarum* to see whether depletion of this enzyme imposed an intolerance for H_2O_2 or for aerobic conditions. We now report such experiments which demonstrate that the Mn catalase is indeed important for the aerobic survival of *L. plantarum*.

MATERIALS AND METHODS

L. plantarum strains 8014 and 14431 were obtained from the American Type Culture Collection, Rockville, Md. The former is Mn catalase negative and the latter is Mn catalase positive (14a). Both strains can produce a heme catalase when grown in the presence of hemin (12). Lyophile cultures were subcultured onto multiple APT (9) agar slants which were stored at −70°C until used. A fresh agar slant was thawed and used for each experiment. Cultures were routinely grown on commercial (BBL Microbiology Systems, Cockeysville, Md.) APT broth or agar at 37°C. Growth in liquid medium was monitored in terms of absorbance at 600 nm. Viable cells were enumerated by dilution with sterile 0.9% NaCl, followed by plating onto APT agar and counting of colonies after 18 h of incubation. Aerobic liquid cultures were shaken at 140 rpm, whereas anaerobic growth was done in a Coy Chamber (Coy Laboratory Products, Inc., Ann Arbor, Mich.) or as described by Balch et al. (5).

Liquid cultures were grown in Erlenmeyer flasks with a vessel/fluid volume ratio of 5:1. Cells were collected by centrifugation at 6,090 × *g* for 5 min, washed by resuspension in APT salts, and then resedimented at 6,090 × *g*. APT salts contained (per liter) 5.0 g of K_2HPO_4, 5.0 g of NaCl, 1.25 g of Na_2CO_3, and 0.8 g of $MgSO_4$, adjusted to pH 6.7. Washed cells were

finally suspended in 0.1 M potassium phosphate–0.1 mM EDTA at pH 7.0 and were fragmented by passage through a French press (Aminco) at 20,000 lb/in^2. The resultant homogenate was clarified for 20 min at 17,300 × *g*, and the clear extract was dialyzed against the cold neutral phosphate-EDTA buffer for 24 h before assay. Protein was measured by the Lowry procedure (15), whereas catalase was assayed by the method of Beers and Sizer (6) with slight modification (14a).

$NH_2OH \cdot HCl$ (Aldrich Chemical Co., Milwaukee, Wis.) was dissolved each day and adjusted to pH 6.0 with 1.0 M NaOH before use. Puromycin was from Sigma Chemical Co., St. Louis, Mo. When NH_2OH and puromycin were to be added to suspensions of cells they were sterilized by ultrafiltration. Crystalline catalase from bovine liver and bovine hemin (type 1) were from Sigma. Catalase and hemin, which was dissolved in 50 mM K_2HPO_4 before use, were also sterilized by ultrafiltration.

RESULTS

Effect of catalase on growth and survival of *L. plantarum*. Anaerobically grown inocula of Mn catalase-negative (ATCC 8014) and Mn catalase-positive (ATCC 14431) strains of *L. plantarum* were seeded into aerobic APT broth, and growth was monitored in terms of turbidity. Under aerobic conditions the Mn catalase-negative strain actually grew somewhat more rapidly and to a 20% greater plateau density than did the Mn catalase-positive strain. This agrees with earlier work (17) and fails to demonstrate any advantage imparted by the Mn catalase.

When survival of cell suspensions during the stationary phase was measured, a strikingly different picture emerged (Fig. 1). The viability of the Mn catalase-positive strain (line 3) was well maintained, whereas that of the Mn catalase-negative strain (line 6) plunged down seven orders of magnitude. This loss of viability of the Mn catalase-negative strain was diminished anaerobically (lines 4 and 5). Under aerobic conditions addition of bovine liver catalase, to 100 μg/ml, completely protected the Mn catalase-negative strain (line 2). When hemin is present in the medium these organisms can incorporate it into a true catalase, and, as shown by line 1, growth in the presence of 10 μg of hemin per ml also protected the Mn catalase-negative strain.

Since the moderate loss of viability seen under anaerobic conditions (line 4) was not prevented by 100 μg of catalase per ml (line 5), we conclude that oxygen exclusion was effectively total and that this loss of viability was not due to H_2O_2 lethality. The culture medium in which the Mn catalase-negative strain had grown accumulated H_2O_2 to 8.8 mM (1, 14a). No H_2O_2 was detectable when hemin or catalase was present in the growth medium of the Mn catalase-negative strain. The Mn catalase-positive strain did not accumulate H_2O_2 (14a). It thus appears that

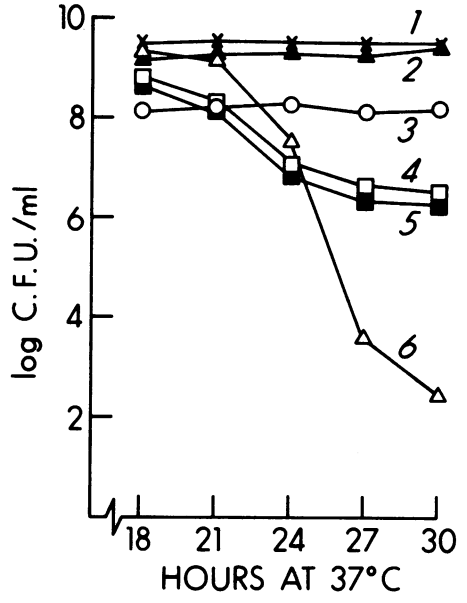

FIG. 1. Survival of catalase-positive and catalase-negative strains of *L. plantarum*. Ryan flasks of APT broth were inoculated (1% inoculum) with overnight anaerobic cultures and were shaken at 140 rpm and 37°C in air while growth was monitored at intervals in terms of absorbance at 600 nm. Enumeration of surviving cells was as described in the text. Catalase-negative ATCC 8014 was grown without additions (line 6) or in the presence of 10 μg of hemin per ml (line 1) or 100 μg of bovine catalase per ml (line 2). This catalase-negative strain was also grown anaerobically in the presence (line 5) or absence (line 4) of 100 μg of bovine catalase per ml. The catalase-positive strain was grown aerobically without additions (line 3). The catalase-negative strain contained 38 U of cyanide-sensitive catalase per mg of protein when grown in the presence of hemin.

the H_2O_2 produced by these organisms can exert a profound lethality if it is not scavenged enzymatically. The ability to produce an Mn catalase clearly has survival value.

Inhibition of Mn catalase by hydroxylamine. NH_2OH was found to be an effective inhibitor of Mn catalase in the presence, but not in the absence, of H_2O_2. Thus, incubation of the enzyme with either 4 mM ascorbate (line 1) or 10 μM NH_2OH (line 2) had no effect, but incubation with both (line 3) caused progressive inactivation (Fig. 2). The aerobic ascorbate solution was used here as a continual source of H_2O_2, as it has previously been used by other workers (16). The importance of H_2O_2 for the NH_2OH inactivation of the enzyme was verified by dialysis of Mn catalase plus 50 μM NH_2OH against either buffer or against 2 mM H_2O_2 in the buffer. In the former case there was no loss of activity,

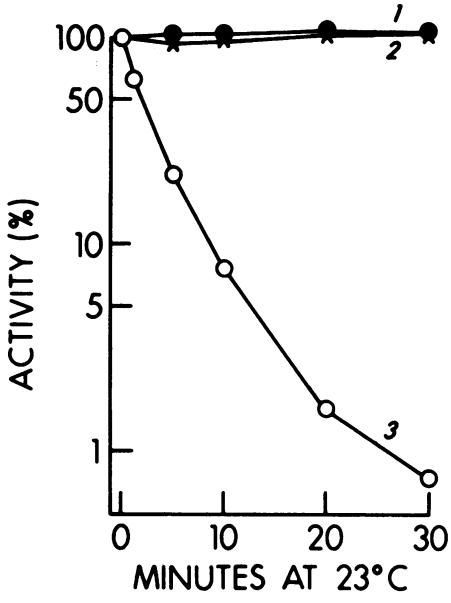

FIG. 2. Inactivation of Mn catalase by hydroxylamine plus ascorbate. Mn catalase, isolated as previously described (14a) and at 51 nM, was incubated aerobically at 23°C in a water bath shaker at 200 rpm, in 50 mM potassium phosphate (pH 7.0), in the presence of: 4.0 mM ascorbate (line 1), 10 μM NH_2OH (line 2), or both (line 3). The reaction volume was 1.0 ml. At intervals 100-μl samples were diluted into 2.9 ml containing 16.7 mM potassium phosphate, 19.7 mM H_2O_2, and 0.1 mM EDTA at pH 7.0. Consumption of H_2O_2 was followed at 240 nm.

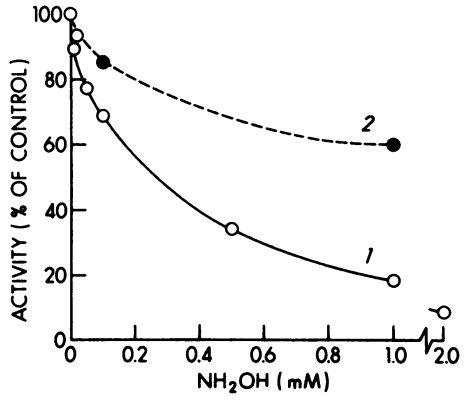

FIG. 3. Inactivation of Mn catalase within *L. plantarum* by NH_2OH. *L. plantarum* (ATCC 14431) was grown aerobically to late log phase (12 h) in APT broth and was then diluted fivefold (to absorbance at 600 nm = 0.1 to 0.15) into fresh APT broth containing the indicated concentrations of NH_2OH. After 2 h of aerobic (line 1) or anaerobic (line 2) incubation at 37°C, extracts were prepared and assayed for protein and for catalase activity. Activity is given as a percentage of control values. The control values were 100.2 U of catalase per mg of protein for the aerobically grown cells and 87 U of catalase per mg of protein for the anaerobically grown cells.

whereas in the latter complete inhibition was seen within 5 min.

The H_2O_2-dependent inactivation of Mn catalase by NH_2OH was not reversed by dialysis and was not prevented by 0.21 μM superoxide dismutase or 10 mM mannitol. It was observed above, but not below, pH 6.0. All of this suggested that NH_2OH might be used to suppress the Mn catalase within *L. plantarum* and thus facilitate demonstration of its physiological importance.

Effects of NH_2OH on *L. plantarum*. *L. plantarum* cells were grown to late log phase (12 h at 37°C), either aerobically or anaerobically, in APT medium. These cultures were then diluted fivefold into fresh APT medium containing the indicated concentrations of NH_2OH (Fig. 3) and, after 2 h of further incubation either aerobically (line 1) or anaerobically (line 2), the cells were harvested, washed, and lysed, and the extracts were assayed. At any given concentration of NH_2OH there was more inactivation of Mn catalase aerobically than anaerobically. This is in accord with the aerobic production of H_2O_2 by *L. plantarum* and the H_2O_2 dependence of the NH_2OH inactivation of Mn catalase. It seems possible that the modest inactivation seen anaerobically was due to our failure to completely exclude O_2 in this case.

The inactivation of Mn catalase within *L. plantarum* by NH_2OH was not accompanied by rapid loss of viability, and removal of NH_2OH resulted in regain of catalase activity, presumably by resynthesis (Fig. 4). In these experiments the Mn catalase of *L. plantarum* was suppressed by aerobic incubation for 2 h in APT medium containing 0.5 mM NH_2OH. The cells were then chilled and washed twice in cold APT medium to remove NH_2OH. The cells were then suspended in prewarmed APT medium and incubated aerobically. At intervals cells were collected, washed in cold APT salts, lysed, and assayed for catalase activity. The specific activity of Mn catalase in aerobically grown cells was 100 U/mg of protein before treatment with NH_2OH. It is clear from Fig. 4 that nearly full activity was restored during 3 h of incubation after removal of the NH_2OH.

L. plantarum, whose Mn catalase was inactivated by treatment with NH_2OH, exhibited an intolerance towards H_2O_2 (Fig. 5). These cells were grown aerobically for 12 h in APT medium and were then diluted fivefold into fresh medium containing 0.0, 0.1, and 1.0 mM NH_2OH. After 2 h of aerobic incubation, cells were collected by

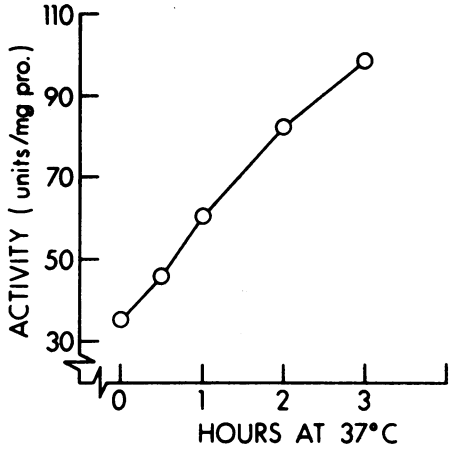

FIG. 4. Recovery of Mn catalase in *L. plantarum* after inactivation by NH_2OH. *L. plantarum* (ATCC 14431) was grown aerobically for 12 h at 37°C in APT broth. The pseudocatalase was then inactivated by aerobic incubation for 2 h in fresh APT containing 0.5 mM NH_2OH. The cells were then washed twice in cold APT medium to remove NH_2OH and then allowed to resume growth in prewarmed (37°C) APT broth. At intervals cells were removed for assay of catalase activity.

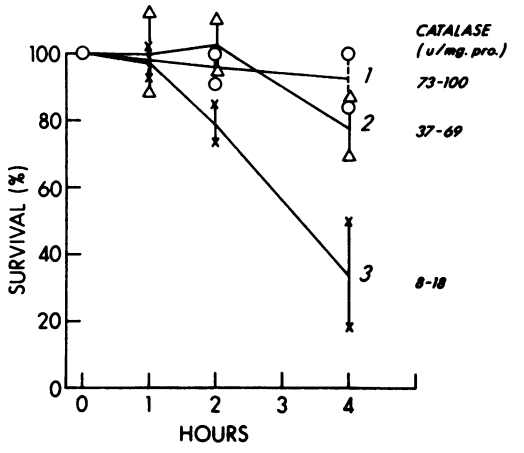

FIG. 5. Inactivation of Mn catalase with NH_2OH sensitizes *L. plantarum* towards H_2O_2. A culture (ATCC 14431) was grown aerobically for 12 h in APT broth. Samples were then diluted fivefold into fresh APT containing 0 (line 1), 0.1 mM (line 2), and 1.0 mM (line 3) NH_2OH. After 2 h of aerobic incubation, cells were collected, washed twice in cold APT to remove NH_2OH, then suspended to 10^8 cells per ml in fresh APT containing 5.0 MM H_2O_2 and 0.5 mg of puromycin per ml to prevent protein synthesis. These suspensions were incubated aerobically at 37°C, and samples were taken at intervals for enumeration by dilution, plating, and counting. The numbers given to the right of the lines are the levels of Mn catalase activity found in these cells.

centrifugation, washed twice in cold APT, and then suspended to 10^8 cells per ml in APT containing 5 mM H_2O_2 and 0.5 mg of puromycin per ml to prevent resynthesis of Mn catalase. These suspensions were incubated aerobically at 37°C. At intervals samples were taken, diluted, and plated for enumeration of viable organisms. Cells whose Mn catalase was not suppressed by NH_2OH were not noticeably killed by 5 mM H_2O_2 plus puromycin (line 1), whereas those treated with 0.1 (line 2) or 1.0 (line 3) mM NH_2OH exhibited a progressive intolerance towards H_2O_2. The failure of those cells which had not been treated with NH_2OH (line 1) to grow in the APT medium, containing 5 mM H_2O_2 and 0.5 mg of puromycin per ml, is an indication that this level of puromycin was sufficient to suppress protein biosynthesis. Previous studies have shown that 0.5 mg of puromycin per ml is sufficient to completely prevent biosynthesis of catalase, peroxidase, and superoxide dismutase in *Escherichia coli* (10).

DISCUSSION

The Mn catalase-negative strain of *L. plantarum* does grow slightly more rapidly aerobically and achieve a somewhat higher cell density than does the Mn catalase-positive strain. This is not really too surprising since production and accumulation of H_2O_2 by the Mn catalase-negative strain are not noted until the late log and stationary phases of growth (1, 14a). However, when cell survival during the stationary phase was examined, the advantage provided by Mn catalase was obvious. NH_2OH, which caused an irreversible inactivation of Mn catalase in the presence of H_2O_2, allowed this demonstration to be made with the Mn catalase-positive strain. Suppression of Mn catalase, within these cells, rendered them sensitive to the lethality of 5 mM H_2O_2. The biological utility of Mn catalase is thus clearly demonstrated.

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